

SCIENTIFIC WHITE PAPER

Advanced Analytical Approaches for Accelerated Development of Gene Therapy Products

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Advanced Analytical Approaches for Accelerated Development of Gene Therapy Products

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Advanced therapeutic platforms like gene and cell therapies offer tremendous potential for treating unmet medical needs but are extremely challenging to develop successfully, largely because their molecular platforms and clinical delivery systems are highly complex. Obtaining a better understanding of specific structural attributes that influence clinical delivery and outcomes is essential for advancing both individual gene therapy candidates, as well as the emerging vector/gene platforms on which they are based. In particular, identifying and controlling specific molecular attributes of a vector, quantifying vector delivery to the target therapeutic site and correlating therapeutic protein expression *in vivo* with clinical response are all now beginning to emerge as important strategies and underpinnings in leading gene therapy development programs. Concurrently, advances in high-resolution LC-MS based vector protein characterization, *in situ* quantification and *in vivo* expression profiling approaches are increasingly being applied to enable improved program understanding, controls and clinical outcome analysis to support both development and potentially commercialization. This white paper describes leading approaches in detailed characterization and quantitation of gene therapy vector proteins, delivery to target tissues and therapeutic protein expression through development and into regulatory packages. It conveys how understanding the relationship between specific molecular attributes identified through high-resolution analyses and quantification of related *in vivo* expression influences efficacy and can guide, de-risk and improve development of complex gene therapy products. The primary focus here is on AAV systems with inclusion of related aspects of LVV; however, the methods reviewed may be applied to other related platforms for advanced therapeutic medicinal products as well. Specifically, this paper presents advanced LC-MS based analytics and examples of how these approaches are being applied to elucidate attributes of vector proteins that are critical to efficacy and quality.

Introduction

We are currently experiencing unprecedented innovation, growth and opportunity within the biopharmaceutical industry, with increasingly promising yet complex biologic therapeutic platforms in development, including diverse gene therapy approaches. Despite significant advances in therapeutic platform development, almost 90% of biologic drug candidates fail from Phase I to Approval,¹⁻³ with over 50% of biologics continuing to fail in Phase III. These challenging trends are even more pronounced in the highly complex systems of gene and cell therapy development. Moreover, the substantial costs of developing advanced therapeutic medicinal products (ATMPs) coupled with the high risk of failure, not only in obtaining approval but also post-approval with the increasing need for outcome-based pricing,⁴ makes it increasingly important for innovators to better understand, demonstrate and control the drivers of clinical efficacy and success.

Gene therapies utilize an array of complex delivery vehicles to accomplish gene introduction or modulation and expression of therapeutic protein in the patient. Because of the complexity of vectors, as well as their substituents and structures, strategic application of analytical approaches is increasingly important in the successful development of gene and cell therapies. Accordingly, in an effort to gain and demonstrate improved understanding and control of these complex products, leading groups have begun integrating higher resolution analytical characterization approaches throughout the development process. These studies include detailed analysis of the vector constructs, delivery systems and *in vivo* expression products. This evolution can be likened to the maturation of analytics and bioproduction in monoclonal antibody (mAb) development. Since the early days, addition of higher resolution techniques has led to now well-established analytic platform approaches. Advanced analytics have resulted in well-characterized mAb products and provide production information to guide process

development. They further ensure reliable control of manufacturing of quality mAb products through development and in commercial production. The evolution was also in part successful due to an expanded repertoire of increasingly sensitive and high-resolution analytical tools being used to assess attributes of numerous molecules to identify common features that influence biological functions as well as product quality. More recently, by interrogating molecular details of biologic therapeutics once administered to patients, identification of *in vivo* critical quality attributes (CQAs) can be acquired to advance understanding of attributes that impact therapeutic success.

As with mAbs and other complex biotherapeutic platforms today, high-resolution analytic approaches are similarly being integrated into gene therapy vector development as well as assessments of delivery to target tissue and clinical program designs to identify specific attributes of gene and cell therapy systems that can drive successful vector design, selection and production through clinical efficacy.

Concurrently, the thinking of both developers and regulators is evolving to include the use of such detailed molecular analyses at the platform and delivery, protein expression and clinical outcome levels. Particularly, a few recent examples, as described in this article, have been reported that show how advanced LC-MS (liquid chromatography mass spectrometry) approaches have been deployed to inform, guide and control such development at the vector, target delivery and *in vivo* expression level. These studies begin to demonstrate how understanding gained by investigations with high-resolution techniques offers tremendous potential for improving processes and designs of complex therapeutics to enable more efficient development of gene and cell therapy products.

Overview of Gene Therapy

Gene therapy as a broad classification is a rapidly growing area of therapeutic development that includes a variety of complex delivery vehicles ranging from viral vectors to modified cells to nucleotides and gene editing enzymes in complex formulations.

Early gene therapies utilized adenovirus for gene delivery because the use of a replication competent viral vector was considered necessary for persistence of a therapeutic effect. In a clinical trial in 1999 a fatal immune response to this adenoviral vector led to multi-organ failure and brain death in a young patient; not only ending the trial, but also setting back the field considerably.

Subsequent scientific investigations led to better understanding the potential clinical risks in administration of gene therapies, and in 2012, the European Medicines Agency (EMA) approved the first gene therapy product Glybera (uniQure). The advantage of this product is that it is based on adeno-associated virus (AAV), an unrelated and self-replication incompetent vector. Glybera led the way in accomplishing successful development of a safe product; however, due to lack of prescribing, attributed to the extremely high price point (>€1M) and insufficient demand, it was consequently terminated post-market. The fate of Glybera and ability to demonstrate efficacy reflects a real concern for companies developing gene therapies, particularly for rare diseases where there are small numbers of patients, as clinical outcomes must be sufficiently good to both entice prescription of the product, and to overcome risks of outcomes-based payment. These early setbacks have provided better foundations and roadmaps for further success ahead.

The amount of investment in this space is also reflective of the increasing promise these gene therapy platforms hold for the industry. In 2013-2018, nearly 240 public funding instances totaling \$9.5B occurred, and over \$9.8B was invested in

gene therapy research by government and private capital sources to fuel the development pipeline.⁵ Since 2017, investment has increased substantially.⁶ In the last two years, large pharma/biotech has spent over \$125B on acquisitions of cell and gene therapy companies.⁷ Many IPOs have also occurred recently as well, with the largest coming in at \$604M for Moderna in December 2018, surpassing the prior raise that October by Innovent of \$421M.⁸ In June 2019, six gene therapy companies went public, bringing in an aggregate investment of nearly \$880M.⁹

Recently, several diverse types of gene therapy products have also been approved by FDA, including:

- ◆ AAV gene therapies Luxturna for night blindness (Spark) and Zolgensma (AveXis/Novartis) for spinal muscular atrophy (SMA)
- ◆ Antisense DNA therapy Spinraza (Biogen) for SMA and RNA-based therapy Exondys 51 (Sarepta) for Duchenne muscular dystrophy (DMD)
- ◆ Autologous cell therapies utilizing CAR-T technology (Kymriah, Yescarta)
- ◆ Oncolytic viral therapy Imlygic (Amgen) for treating cancers.

Many more cell and gene therapies are in development, and most of these candidates are aimed at targeting cancer (~70%), with the remainder focused on treatment of cardiovascular, infectious and rare diseases.

The vast majority of gene therapy studies are in early phases: 75% Phase I, 20% Phase II, less than 5% Phase III,¹⁰ reflecting the recent emergence of these therapeutic approaches. While there are common categories of approaches, a diverse set of delivery vehicles are under investigation in the clinic, including:

- ◆ Viral-based vectors, primarily AAV with some adenovirus

- ◆ Cells transfected with lentiviral vectors (LVV), most often to generate CAR-T therapies
- ◆ CRISPR/Cas9 gene editing technologies
- ◆ Nucleotides, primarily mRNA delivered via liposomal formulations.

Understanding and controlling the critical attributes and production processes that determine clinical success in these complex biologic systems is important for reducing risk in both clinical outcomes and CMC aspects of development. Even within a category, we are just beginning to identify attributes of these products and the corresponding biology that determine their efficacy and safety.

AAV is the most commonly used approach for gene therapy products currently in development.^{11,12} AAV forms an enclosed spherical capsid structure comprised of three variants of a viral protein (VP1, VP2, VP3) that encapsidate a gene up to approximately 5 kb in size (**Figure 1**).¹³

The advantages of AAV are that it has a favorable safety profile in that it does not cause disease in humans, does not integrate into the genome (*i.e.*

stored as episomal DNA in the nucleus) and does not replicate in the patient. In fact, AAV requires assistance from helper virus to be replicated during manufacture of the therapeutic product, conferring safety of this technology. In addition, AAV exists as a diverse set of serotypes, with tropisms for different tissues in the human body. This natural diversity permits some degree of tissue-specific targeting that can be beneficial for treating specific indications, and additionally may be coupled with tissue-specific promoters to better target expression to the intended tissue.

The drawback with most AAV-based therapeutics is that administration is limited currently to a single dose because approximately 90% of the population has prior exposure to the virus, resulting in pre-existing anti-drug antibodies (ADA) that can reduce efficacy of an initial dose and promote further immune reactions, which prevent subsequent dosing.¹⁴ For some serotypes, the prevalence of pre-existing antibodies may be as high as 60% but is variable based on geographic location. For AAV5, ADA as low as 3.2% has been reported but in some regions may be as high as 40%. Interestingly, capsid switching to another AAV serotype to deliver a second dose has been demonstrated to provide substantially greater

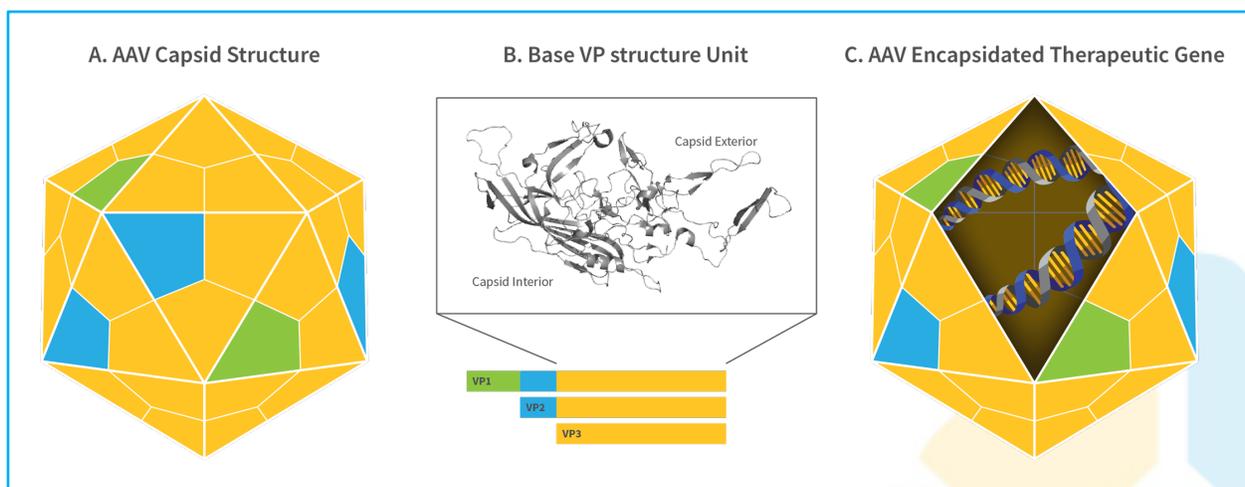


Figure 1. AAV Capsid Assembly and Structures. Panel A shows the assembly of viral proteins VP1 (green), VP2 (blue) and VP3 (gold) as 1:1:10 in the icosahedral capsid structure. Each VP has a core folded unit in common, as shown in Panel B, with the N-terminal extension for VP2 and VP1 shown in blue and green, respectively. The corrected therapeutic gene to be delivered is packaged inside the capsid core (<4.8 kb), as is visible through the removed subunits in Panel C.

expression levels of therapeutic in animals.¹⁵ Re-engineering efforts are being pursued to improve both tissue-specific targeting and immune avoidance, as a single dose is insufficient for full therapeutic effect in some disease states. Tissue uptake appears to depend on the capsid sequence and post-translational modifications (PTMs), whereas the immune response that limits dosing may be highly dependent on the nucleotide sequence and interaction with TRL9.¹⁶ Modification of both vector components, the protein capsid and the nucleotide cargo, are commonly performed to achieve more effective delivery of AAV-based therapies.

Production of AAV gene therapy products is complex. Three major approaches to production are used to generate the desired therapeutic vector product in a safe and effective manner, including a multi-plasmid transfection approach with helper virus, baculovirus in insect cells or stable cell lines with essential components for replication (*rep* and *cap* genes) integrated into the host genome.¹⁷⁻²⁰ Most commonly, triple transfection is used to introduce the functional components of the replication machinery (*rep*, *cap*, etc.) required for production of therapeutic vector product into host cells. Key components are separated because the greatest concern with viral vectors is potential delivery of wild-type infectious virus and dividing these components into separate plasmids significantly reduces the possibility of reforming an infectious wild-type particle. Additionally, the replication machinery is further engineered to disable function of the wild-type virus in the event recombination occurs.

Another prominent approach involves use of retroviral vectors to transfect host cells with a specific gene. Although these vectors have been pursued as a direct gene therapy approach, *ex vivo* construction of genetically modified cell therapy is much more common, as with CAR-T in Kymriah and Yescarta. Lentiviral vectors (LVVs) are most common and have the advantage of being non-immunogenic and also can deliver larger, more complex genes than AAV. Safety concerns,

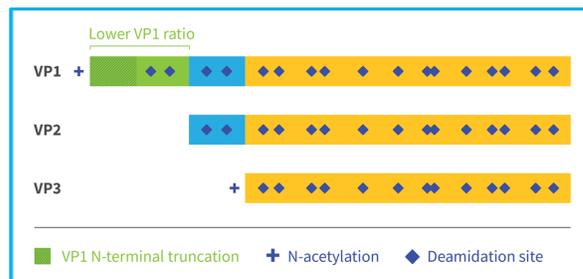


Figure 2. Attributes of AAV that Affect Cellular Uptake and Transduction. Viral proteins VP1, VP2 and VP3 are depicted with potential PTMs that have been identified as having an impact on efficacy and quality of AAV gene therapy products. VP3 (gold) region is common to all VPs. N-terminal extensions of VP1 and VP2 are green and blue, respectively. Cross-hatching represents potential truncation. Acetylation of the N-terminus is shown as + and deamidation as diamonds.

however, are a factor in development of these therapies, and can be better managed *ex vivo*. Retroviral vectors pose a safety risk because they integrate into the genome of patients. This integration facilitates long-term expression, but there is a lack of control over both the tissue into which the gene is delivered and the site of integration into the genome. Consequently, the approach has potential to disrupt the function of normal genes by insertional mutations. The risk is considered reasonably acceptable with regard to therapies for severely ill patient populations, but further control and understanding must be acquired before these approaches may be adapted to treat less severe diseases and conditions.

Key Structure Attributes of AAV and Functional Significance in Drug Development

Efficacy of complex gene therapy products depends on multiple factors, including the vector/protein capsid itself, the efficiency of delivery to target tissue and subsequent expression of the therapeutic protein *in vivo*. The first critical steps in determining effectiveness of an AAV-based gene therapy is in establishing the degree of functional gene incorporation into capsids (infectious titer) and how effectively the vector product enters the cell (infectivity). It is well

understood that AAV viral protein platforms infect cells through structure-based binding of the capsid to available cell surface receptors, where productive interaction of the viral proteins (VPs) with the cellular receptor results in internalization of the capsid particle; this is followed by intracellular trafficking of the gene to the nucleus (transduction) for further processing to permit expression of the desired gene product. Infectivity has been shown to depend on a few factors, including vector/protein structure and ratio of VPs as well as PTMs of the VPs themselves (**Figure 2**). Effective trafficking and further processing by intracellular machinery are required for expression and these processes also have been reported to rely on specific molecular attributes of the vector capsid and encapsidated gene.

A composite structure of AAV2 was reported that revealed the capsid to be arranged as a 60-protomer icosahedron of VP1:VP2:VP3 in a 1:1:10 ratio, with C-termini facing outward (see **Figure 1**).¹³ The VP ratio has been observed to vary,²¹ and recent experimental results by capillary gel electrophoresis show VP1:VP2:VP3 to be present at 1:2:7. Analysis of several different AAV therapies by our team also showed variation in the ratio of VPs, further indicating that the ratio of viral proteins in an AAV capsid can deviate significantly from the previously reported structure. It has been suggested that this variance may depend on differences in the capsid protein sequence that correspond to serotype, making it important to determine for each system, particularly because the N-terminal region of VP1 has been shown to be important for infectivity and transduction.^{22,23}

Analytical Approaches Used in Development of AAV Gene Therapy Products

Gene therapies are more complex than traditional biologics because they are comprised of multiple proteins encapsidating a gene, which makes them more challenging to *well characterize* at a level consistent with other biologic drugs today. As such, many optimized product-specific analytical

Table 1. Analytical Approaches Used in Development of Gene Therapies

Category	Attribute	Methods
Therapeutic Gene	Identity	Sequencing, gel electrophoresis
	Viral titer	qPCR
Viral Capsids	Contents (full, partial, empty)	Centrifugation/AUC, TEM, MS
	Viral protein (VP) ratio	HPLC, CE, SDS-PAGE, Western blot, MS
	VP integrity (PTMs/degradants)	HPLC, IEX chromatography, MS
	Size/aggregation	Fluorescence (DSF), light scattering, AUC
Purity	Host cell proteins (HCPs)	SDS-PAGE, ELISA, MS
	Host cell DNA/RNA	PCR (qPCR, ddPCR)
Potency	Infectivity	Bioassays (cell culture, animal)
	Quantitation of translation of therapeutic gene (in vitro, in vivo)	Western blot, MS
	Activity of gene product	Biochemical assay (enzymatic, clotting, etc.), clinical outcomes

techniques and approaches are required for the successful development of gene therapies. **Table 1** outlines core categories and attributes currently analyzed in development of an AAV gene therapy, and **Table 2** further details aspects relevant to application of each technique.

Analysis of Gene and Nucleotide Components

Gel and capillary electrophoresis are rapid approaches to confirm the presence and correct size of the nucleotide/gene incorporated into the therapeutic product. PCR-based methods provide more specific quantitation and confirmation that the intended gene has been incorporated. Sequencing is performed to verify the fidelity of the full gene sequence and flanking regulatory elements.

A critical feature of the therapeutic product is viral titer, because the quantity of virus is often the basis of dosing. qPCR is performed using primers targeting the viral genome to establish the amount of gene present in the product.²⁴ To further guide dosing, well developed bioassays are key to establishing infectivity and infectious titer of a product. Additionally, comparison of the viral titer with the amount of total viral protein can provide an indication of the amount of full vs empty capsids in the product, a ratio that can vary greatly and has been reported to impact efficacy.

qPCR is also routinely conducted for detection of specific potential viral contaminants. The method is applied using primers that target other specific

Table 2. Techniques for AAV Vector, Viral Protein (VP) and Capsid Characterization

Category	Attribute	Methods	Advantages	Disadvantages and Considerations	
Therapeutic Gene	Identity	DNA sequencing	Confirms identity of full nucleotide sequence	Poor sensitivity for low abundance sequence variants	
		Electrophoresis (gel, capillary)	Fast, estimates size and amount of gene in sample	Not specific to gene of interest or sequence variations	
	Viral titer	qPCR	Accurate, selective quantitation of gene of interest	Requires consistent cells growth, sample handling, high-quality primers and reference dye for reproducible results	
Viral Capsids	Capsid & contents (full, partial, empty)	AUC	Separation based on density. Widely applicable	Works for only some AAV. Inefficient, small scale. Small differences may be difficult to interpret	
		TEM	Widely applicable, straightforward to interpret	Inefficient, small scale. Potential surface-induced artifacts	
		MS	Fast, amenable to large scale, distinguishes partially full	Expertise & specialized instrumentation required	
	Viral protein (VP) ratio	HPLC	Fast, easy to perform, quantitative	Potential co-elution of modified species	
		CE	Fast, easy to perform, quantitative	Pattern may be complex and difficult to interpret	
		SDS-PAGE	Fast, easy to perform	Pattern only. Couple with Western blot or MS	
		Western blot	Fast, relatively easy to perform	Prone to false positives/negatives, inexact quantitation	
	VP integrity (PTMs/degradants)	MS	Fast or provides information on PTMs	Expertise & instrument required	
		HPLC	Fast, easy to perform, quantitative	Potential co-elution of modified species	
		IEX chromatography	Fast, easy to perform, quantitative	Works for only some AAV	
	Size/aggregation	MS	Provides ID and PTMs, low level quantitation	Expertise & instrument required	
		Fluorescence (DSF)	Widely applicable, fast, robust, stability indicating (T_m)	Exogeneous dye used. T_m sensitive to conditions	
		Light scattering	Fast, easy to perform	Small differences may be difficult to interpret	
	Purity	Host cell proteins (HCPs)	AUC	Widely applicable, large size differences	Inefficient, small scale
			SDS-PAGE	Fast, easy to perform	Pattern only. Couple with ELISA or MS
ELISA			Fast, relatively easy to perform, quantitative	Custom assay & reagents. Potential for false negative/positive results. Inexact quantitation	
MS		HCP ID, quantitative, robust, avoids problems of ELISA	Custom assay, expertise & instrument required		
Residual DNA/RNA		qPCR	Accurate quantitation of viral replication genes, process/product-related species, adventitious agents	Primer design requires prior knowledge of potential contaminants	
Potency		Infectious titer	Bioassay + detection of gene	Reports on activity as a function of total capsid and viral titer to aid in dose determination	Combines two assays. Model system must fully emulate properties of human biology to yield meaningful results
	Quantitation of gene product (in vitro, in vivo)	Western blot	Fast, relatively easy to perform	Dilutional non-linearity, inexact quantitation, prone to false positives/negatives	
		MS	Quantitative, CQAs, avoids problems of Western blot	Custom assay, expertise & instrument required	

gene sequences that correspond to regions of potential concern, such as those from the helper plasmid DNA used for manufacture, to ensure the vector is substantially free of process- and product-related impurities.²⁵ For example, it is critical to determine the *rep* gene is not detected down to the very low limit allowed by regulators to ensure safety.

Analysis of Capsid Viral Proteins

SDS-PAGE, Western blotting and HPLC have been used to monitor the proteinaceous components, including VP1, VP2 and VP3 as well as their relative ratios. While these are straightforward analyses to conduct in theory, it is common that additional bands/peaks appear in gels, suggesting altered VP forms and/or additional impurities may be present

(Figure 3). In some LC separations, elution/migration position of a VP can be altered by degradation or modification and may result in overlap between peaks, confounding results.

An example was reported for AAV5 wherein VP2 and VP3 initially were resolved peaks but upon stability testing the relative intensity of the peaks changed. To determine the basis of this change, mass spectrometry (MS) analysis was used to identify the species present in each peak. The MS analysis showed that deamidation of VP3 led to a shift in retention time and that the deamidated form co-eluted with VP2 such that the peak observed by UV with increased intensity contained both VP2 and deamidated VP3.²⁶ Being able to track and differentiate VP2 from deamidated VP3 was important because deamidation was also shown to correlate with a strong decrease in

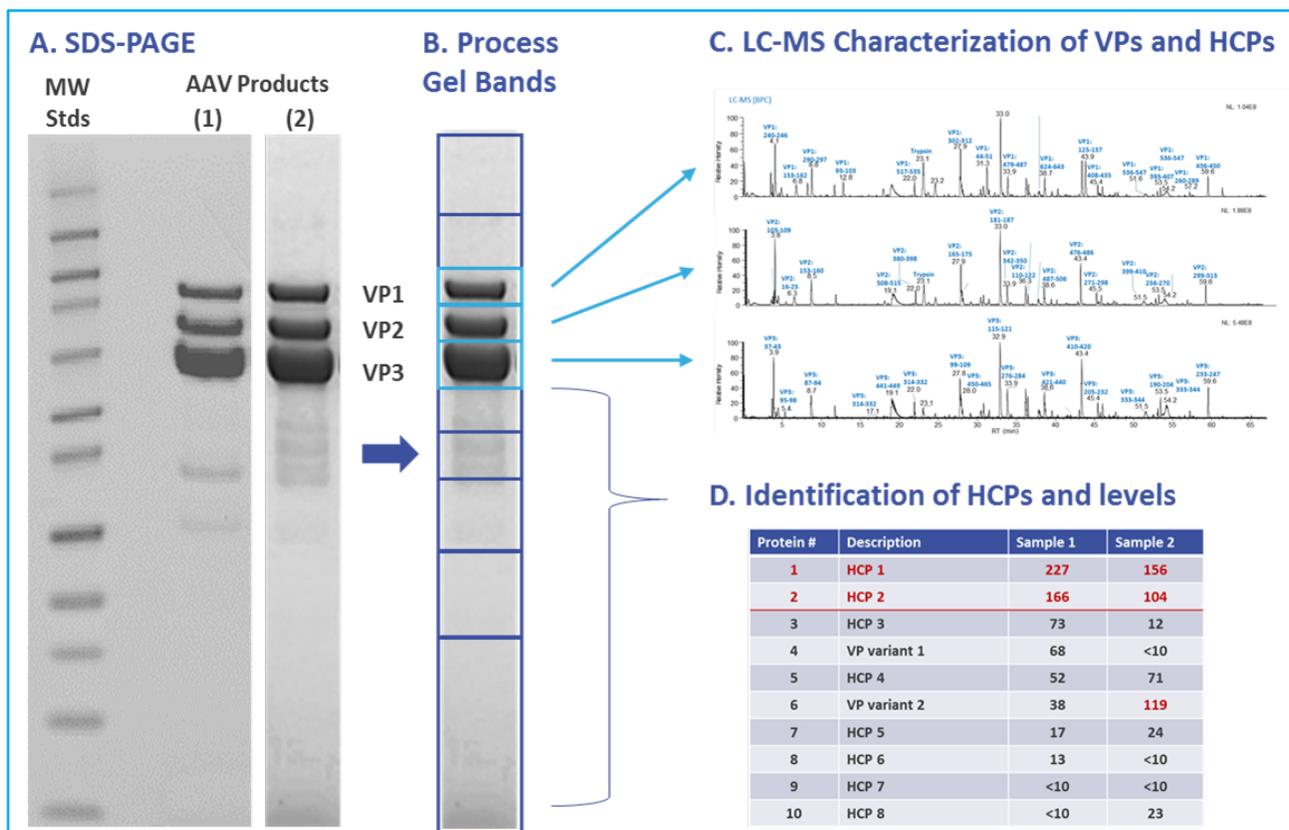


Figure 3. Approach to Characterization of AAV Gene Therapy Products by LC-MS. Panel A shows two different AAV gene therapy products (1 and 2) analyzed using SDS-PAGE. Additional bands are observed along with the expected three VPs. To determine whether the unknown bands correspond to VP variants or unrelated HCP impurities, the gel is segmented, and each slice is processed (*i.e.* digested, extracted; B) and analyzed using LC-MS (C). From the masses observed, each VP, VP variant and HCP species is identified, and the relative level of each then quantified (D). As depicted in Panel D, HCP and VP variant levels of concern are shown in red and those below the red line are considered within an acceptable range.

infectivity and a concomitant loss of potency in cell-based assays for this therapeutic.²⁶ Consequently, data from detailed molecular characterization has provided clear insights about potential efficacy of an AAV candidate or batch to guide candidate selection and process development. MS analysis has been further applied to other gene therapy systems for similar purposes,²⁷ for example to identify unknown bands in SDS-PAGE separations (Figure 3). As shown in Figure 3, additional bands may be extracted and digested from the gel and analyzed using LC-MS to determine if they correspond to VP variants or host cell protein (HCP) impurities.

AAV Vector Protein Characterization Including Co-Produced Host Cell Protein Analysis

In advanced development of AAV gene therapies today, the detailed amino acid sequences and PTMs of the vector proteins are thoroughly analyzed, typically by both intact MS and detailed peptide mapping by LC-MS to confirm identity, including complete amino acid sequence determination/verification, sequence variant analysis and VP ratio analysis. In addition, LC-MS assessment provides site-specific analysis of PTMs and degradation events (stability). Typically, SDS-PAGE analysis followed by in-gel enzymatic digestion and LC-MS/MS is used. However, limited recovery of digested peptides from the gel can

Table 3. AAV Attributes Reported to Impact Activity or Quality

Attribute	Therapeutic Impact	Analytical Approaches
Deamidation	Reduced infectivity	MS
	Decreased capsid stability	DSF
N-terminal truncation of VP1	Reduced infectivity	MS
	Reduced expression	MS
Lower VP1 ratio	Reduced infectivity	HPLC, CE, SDS-PAGE, MS
N-acetylation	Reduced expression	MS
Tyr phosphorylation	Decreased expression	MS, antibody assay
Aggregation	Decreased potency	DSF, AUC

lead, importantly, to incomplete determination of N-terminal sequences of VPs, which is where the three VP variants differ. To ensure characterization of the N-termini, direct LC-MS intact protein analysis can be applied to characterize VPs based on accurate mass measurement, and the approach can additionally differentiate AAV serotypes. LC-MS has been used to confirm N- and C-terminal sequences to verify VP content, confirm the identity of AAV serotype and monitor heterogeneity among capsid proteins as well as detect PTMs.²⁸

As noted above, certain key PTMs have also been reported to impact potency of AAV gene therapy products in development (see **Table 3**). Deamidation of VPs is purported to diminish infectivity of AAV in several systems, and recently the Wilson lab published a study showing the relevance of deamidation to transduction efficiency in diverse AAV serotypes.²⁹ This study revealed 17 sites in WT AAV8 undergo deamidation, some extensively, including N57 and N263. Several sites when mutated to Asp (to mimic degradation) were identified as having >10-fold decrease in transduction efficiency, including N57, N94, N305, Q467, N479 and N653. It was further demonstrated that deamidation increased with time and efficiency of transduction dropped by 60% from day 1 to day 5 for both AAV8 and AAV9. Interpreting the results was complicated, however, by observations that mutation at one Asn could alter deamidation at other sites. It was suggested that N514 and N540 are sites where deamidation could result in decreased transduction by altering receptor binding.^{29,30}

Separately, it was determined that the stability of AAV capsids is decreased with deamidation.³¹ These findings are significant not only with respect to potential serotype differences but also because AAVs contain hypervariable regions that naturally vary in sequence and capsids are often being further engineered, which could alter expected deamidation sites and rates. In this regard, meaningful differences between therapeutic candidates may exist, making it important to characterize each individual candidate.

In addition to deamidation, there are other VP attributes that could affect potency. N-terminal acetylation of VPs was reported to be significant in supporting expression of the gene, as it pertains to intracellular processing and expression of the intended protein.²⁸ Research on other attributes of capsid proteins revealed that basic amino acids play a significant role in trafficking and tissue specific transduction and that sequences specific to VP1 influence intracellular trafficking and entry into the nucleus, making it important to confirm the N-terminal sequence and quantify the amount of VP1.^{22,23} Additionally, phosphorylation of certain Tyr residues on the capsid of AAV2 was observed to occur. Although infectivity was not altered, phosphorylation correlated with significantly reduced trafficking to the nucleus and concomitant lower transduction efficiency.^{32,33}

The understanding that already has been gained about specific attributes that impact quality and efficacy through application of high-resolution LC-MS analyses indicates that the use of these approaches is and will continue to be highly valuable for successfully advancing individual AAV gene therapies through development. As well, the acquired data and resultant insights provide helpful guidance on attributes of the base delivery vehicles being employed for numerous candidates. **Figure 2** shows a generalized representation of AAV VPs depicting the aforementioned PTMs.

While AAV gene therapy candidates have often been observed in a non-glycosylated form,^{28,34}

numerous consensus motifs for glycosylation are present in the sequence, indicating VPs also have potential to be glycosylated. Recently, analysis by mass spectrometry revealed that AAV8 produced in HEK 293 cells was glycosylated at N499.³⁵ As with simpler protein biologics, this is an important aspect of AAV-based product development to profile when relevant, because such PTMs of capsid proteins can have a significant impact on viral properties, especially tropism.³⁵ It is also well established that specific types of glycosylation may reduce immunogenicity of capsid-coated viruses as well as aid their intracellular processing and slow degradation.^{32,33,35} It has further been suggested that glycosylation may impact capsid assembly and influence the ratio of VPs in a particle. As such, differences in sequence and/or expression systems used for AAV production may result in different glycan profiles of VPs, thereby impacting various aspects of efficacy determinants, including tissue targeting, infectivity and gene expression. While many current AAV candidates purportedly lack glycosylation, the potential benefits of these attributes may lead to more glycosylated products going forward.

Profiling Full vs Empty Vector Protein Capsids—Incorporation Analysis

It is necessary to determine the contents of capsid particles, as the percentage of capsids containing the therapeutic gene can vary substantially, potentially impacting resulting therapeutic efficacy in patients. Optimally, all capsids should be filled with the therapeutic gene (incorporated) but many are not, resulting in empty or partially full capsids. For some AAV serotypes, anion exchange chromatography can be used to separate full capsids from empty ones.³⁶ Depletion of empty capsids is more challenging for other types such that even following purification, particles lacking therapeutic gene are present in appreciable amounts. In some cases, this appears to have little impact on therapeutic efficacy, but in others empty capsids can substantially reduce efficacy,^{25,26,36,37} presumably by competing with

full capsids for binding to receptors and reducing uptake into cells. Regardless, percent incorporation is an important factor that should be considered when determining dosage to ensure sufficient amounts of gene are being administered to achieve potential therapeutic efficacy,³⁸ and also because the contents of empty/partial capsids are released inside the cell. As such, the relative proportion of full and empty capsids should be determined, and several analytical approaches have been used for assessment (**Table 1**).

The tool kit for incorporation analysis is large and continues to expand, which reflects the need to obtain better information and more practical approaches for monitoring. In some cases, fast, straightforward determination of percent incorporation has been obtained with chromatography, but this does not apply widely due to differences in overall chemical properties among serotypes. Centrifugation and microscopy approaches are amenable to diverse systems and have been developed to differentiate full and empty capsids based on a difference in particle density. These methods, however, are low resolution, inefficient and impractical for commercial scale testing and controls. For this reason, charge detection MS was developed to quantify full vs empty AAV capsids, and it is further capable of distinguishing the partially full population as well.³⁹ While there may be differences in the approach, Pfizer has reported utilizing MS-based analyses to distinguish empty and full AAV capsids in their large-scale production lots, demonstrating the practical utility of the approach for this purpose.⁴⁰

Profiling Host Cell-derived Impurities in Vector Production

Empty particles are also shown to be not truly empty, but in fact contain a range of potential impurities, including proteins and DNA/RNA species derived from the host cell production systems. Most commonly, HEK293 cells are used for production of AAV vector particles; however,

these gene therapy vector production systems may involve diverse host cell types, including insect, rodent and/or human cells. Each production system allows for introduction of unique host-derived protein and nucleic acid impurities, which pose different potential risks.

As noted above, nucleic acid-based impurities can include not only plasmid DNA but also host DNA/RNA and human viral sequences that may be transmitted through the therapeutic and are monitored using PCR (for a detailed review of DNA/RNA analysis see JF Wright *Biomedicines*, 2014²⁵). Released particles can be harvested from cell culture supernatant, but some systems may require purification from lysed cell extract. The type and abundance of host DNA/RNA and HCPs differ greatly between the two harvest approaches, affecting the potential impurity profile. Residual impurities also can be present outside the capsid compartment, and the purification approach used can impact the number, level and type of HCPs remaining in the purified product.^{25, 27} This holds not just for AAV but also other delivery systems, such as LVV.²²

HCPs can result in immune reactions, potentially leading to safety concerns and/or diminished therapeutic efficacy, which could result through the development of neutralizing antibodies. As such, HCPs must be profiled in these products.⁴¹ Traditionally, HCP quantitation and proteome coverage profiling has been conducted using antibody-based assays, but an MS-based HCP identification and characterization approach is increasingly common, at least as an orthogonal method. As a product moves through clinical development, this is in fact required by regulatory agencies. The MS-based approach not only provides confirmation of identity of individual impurities, but also provides quantification. Very importantly, it is highly sensitive and does not suffer from non-linearity issues or false positive/negative results often seen with antibody-based methods. For MS profiling, a library must be constructed based on the cell source(s) used to generate the material to be

analyzed. The quality of the HCP profile depends on the thoroughness of the library, and moreover, must account for cell line specific differences and changes that occur when the recombinant gene, capsid and/or gene product(s) are produced. The MS approach, involving enzymatic digestion of proteins into smaller peptides, may be carried out in solution and/or first subjected to a gel-based separation to improve detection and for completeness of the assessment (see [Figure 3](#)).

CAR-T cell therapies utilize multiple vectors and may have even more complex HCP profiles. Kymriah and Yescarta use different viruses to accomplish expression in T-cells. Kymriah utilizes LVV, whereas Yescarta uses a gamma retroviral vector. Both viruses use the same proteins for packaging (*i.e.* Gag, Pol and Env), but the isoforms of these proteins differ as do their manufacturing approaches. As a result, CAR-T products could have an array of unique protein coat properties as well as residual HCP impurities,²² which may have different consequences *in vivo*, and therefore, are characterized at the viral vector level.

Quantifying and Profiling Expression of AAV Gene Products *In Vivo*—*In Vivo* CQA Mapping

In addition to obtaining detailed characterization of the AAV vector/capsid constructs being administered as drug product, quantitative analysis of the potential gene expression products *in vivo* can provide critical understanding for controlling delivery of the vector and optimizing transduction/expression of the gene product in the target tissue in the patient. Particularly, quantifying the expressed protein and profiling the critical quality attributes and PTMs that correlate with clinical parameters and/or outcomes can guide dosing as well as selection and optimization of candidates with greater chance of success in the clinic.

Quantitation of Delivery to Target Tissues

Quantification of viral titer of a gene therapy product is essential, as this value is used as a key basis for dosing. With some products, viral titer

has been demonstrated to correlate directly with efficacy, but for others correspondence between viral titer and degree of effect is not seen. The reason for the lack of correlation to effect is often unclear and may be due to a number of factors, including poor infectivity or transduction of vector, immune effects and/or low activity of the expressed gene product due to difference in the PTM profile of the protein *in vivo*. Quantitation of vector and/or expressed protein following administration through a clinically relevant period of time can guide dosing and further enable understanding of underlying mechanisms. For example, with Factor VIII gene therapy, for treatment to be cost effective, the level of protein should remain sufficiently high at the end of an eight-year period to continue to provide clinically meaningful benefit.⁴² In one clinical trial for hemophilia A, the level of Factor VIII has been monitored for three years, and while clinical outcomes remain above the threshold for requirement of additional therapeutic intervention, recent results have shown a drop in the protein level.⁴³ Statistical models indicate the expression level had reached a plateau; nonetheless, the reported finding caused a drop in the developer's stock price, affirming that confidence in long-term clinical benefit must be supported by quantitative monitoring of therapeutic expression over an extended period of time to justify the high cost of these therapeutics.

Quantitation of expression product has primarily involved antibody-based detection in target tissues because these assays are straightforward to perform and can be effective at detecting low levels of expressed protein in serum-derived or biopsy tissue samples. They are relatively fast and inexpensive to implement once validated but developing a robust and accurate assay can be both time consuming and expensive to complete. Importantly with respect to quantitation, there are considerable challenges with developing and deploying antibody-based assays for use in complex biologic systems and target tissues, including non-linearity and interference from background components in serum or tissue-based

samples. For example, antibodies can bind to related species or similar epitopes which may be and often are present at high levels in complex tissue samples (serum, tissue, cell media), and the presence of species or conditions that occlude interaction sites may interfere with detection by immunoassay. Moreover, differences among unique tissues and fluids may require optimization of custom immune-detection reagents and/or sample prep approaches to obtain accurate results. These artifacts must be identified and eliminated to generate a suitable quantitation assay. To help guide development of such assays and to further improve confidence and quantification, LC-MS based analysis is being utilized in development for assessing clinical trial results and potentially can be used in monitoring both delivery and ongoing expression of the target therapeutic over time.

Particularly given recent advances in LC-MS resolution and compatible *in-vivo*-based workflows, LC-MS analyses are equally sensitive for detecting low levels of biologic species, but without suffering from the disadvantages associated with antibody-based detection. MS analysis also provides a larger dynamic range of linearity and very high sensitivity of detection with minimal interference from background components to determine expression level in complex tissue samples to enable correlations with therapeutic effect.⁴⁴⁻⁴⁶ The developed approaches can then be applied to track protein production at various time and concentration points to establish a PK profile and monitor the longevity of therapeutic gene expression in target tissues and *in vivo*, for example to correlate dosing with expression levels and persistence of the therapeutic effect both near and long term in patients.

In Vivo Characterization of Therapeutic Protein Products Using LC-MS

In Vivo CQA Mapping requires a carefully developed, robust workflow for isolating complete expression product from a complex milieu (serum,

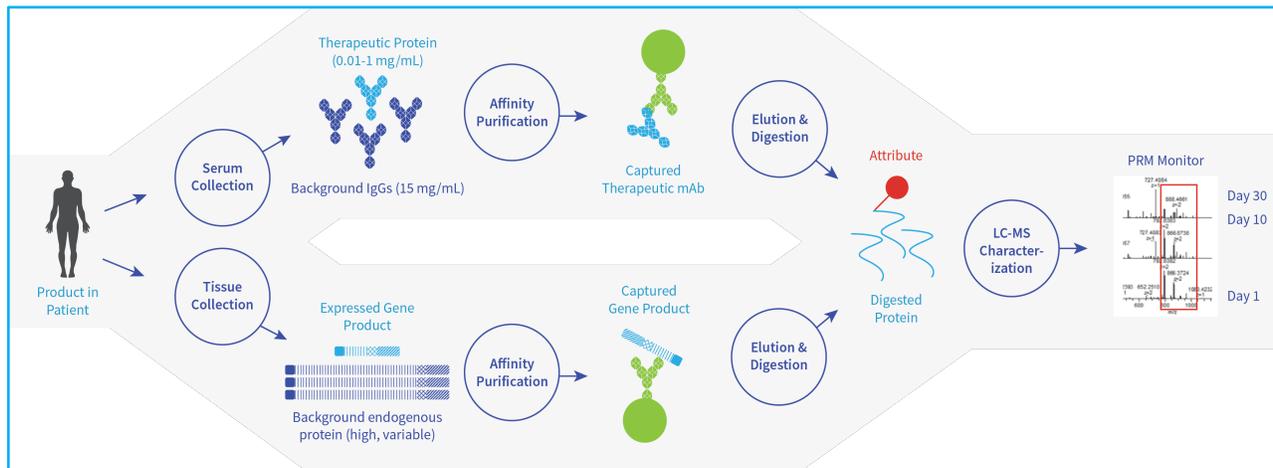


Figure 4. Approach to *In Vivo* CQA Mapping. A schematic cartoon showing example workflow for analysis of a therapeutic mAb (upper) and an expressed gene therapy product (mini-dystrophin; lower) are depicted. Both require selective detection of the therapeutic (light blue) in the presence of substantial background of similar protein (dark blue). Affinity capture (green) of the therapeutic, followed by enzymatic digestion are used to obtain unique peptides for quantitation of the therapeutic and CQA (red) characterization. Data collected from a set of successive time points following administration can then be plotted to assess PK and clinically relevant attributes.

tissue biopsy), followed by enzyme digestion and LC-MS analysis (**Figure 4**).

For therapeutics present in the bloodstream, affinity purification from serum is performed to enrich the protein concentration, which is typically at a low level. In a gene therapy system, some level of endogenous protein is often present along with the expressed therapeutic product, creating substantial background that has potential to interfere with detection and quantitation of the therapeutic, which can be particularly challenging to overcome in antibody-based assays. Isolation of the therapeutic protein or clear identification of peptides unique to each species is critical for accurate quantitation by MS. In addition, biologics typically are heterogeneous and confirming that the isolation approach recovers a true, representative population is important for accuracy of the results as well. MS approaches are able to provide detailed coverage of the molecule to confirm material recovered reflects the therapeutic profile. Still, because of system complexity, use of more than one affinity purification method and appropriate control experiments may often be required to obtain a meaningful assessment. This was demonstrated

with a therapeutic mAb analyzed from both preclinical cynomolgus monkey and human clinical samples,^{45,46} and the approach has been successfully applied to blood-derived samples to quantitatively profile therapeutic proteins expressed from gene therapy products *in vivo* (*unpublished results*). In serum or plasma-based target media, background levels of endogenous protein may complicate antibody-based quantitation, but identification of unique peptides by LC-MS enables a therapeutic expression product to be distinguished from background and accurate quantitation to be obtained. Many recent gene therapies are also being delivered to solid tissues, and the approach to extraction of a gene product must be further adapted to accommodate the additional complexity of a biopsy tissue. Often gel electrophoresis may be used initially to separate and reduce complexity, such that bands/sections may be excised, digested and the peptides extracted for MS quantitation and characterization. In one therapeutic area, aberrant dystrophin expression can lead to DMD, and several gene therapies are being developed to treat this debilitating disease by using AAV vectors to deliver a functional analog of the gene to muscle cells. Endogenous dystrophin is present at

high levels in the tissue, making selective detection and quantitation of the gene product difficult. As such, a MS-based method was developed, utilizing the general approach outlined above to accomplish accurate quantitation of therapeutic,⁴⁴ which is reflective of this approach in tissue-based analysis.

A recent and related example of leveraging the advantage of LC-MS-based assessment in clinical development of AAV-based gene therapy is present in Pfizer's clinical trial for DMD (NCT03362502). PF-06939926 encodes a truncated human dystrophin gene (mini-dystrophin) in AAV9, and the trial uses LC-MS analysis alongside Western blots as a secondary endpoint to measure clinical outcome in terms of expression level of mini-dystrophin in muscle biopsies. With DMD, there is a large background of endogenous dystrophin in the samples, making it challenging to generate antibodies that distinguish well between background protein and the AAV-derived expression product mini-dystrophin. In addition, the amount of background dystrophin in patients is highly variable. Without an established "normal" reference level, it is difficult to ensure linearity of an antibody-based assay across the spectrum of potential patient samples. The MS assay overcame that problem and was able to distinguish between background and mini-dystrophin by tracking unique peptides derived from the mini-dystrophin expression product and background dystrophin separately. The MS approach in this case and more generally adds significant value for quantifying expression *in vivo* because MS allows more accurate quantitation and further differentiates therapeutic-expressed protein from background protein with greater certainty than Ab-based assays.

In addition to quantifying the total amount of therapeutic gene product expressed *in vivo*, MS analysis may provide meaningful insights to profile the attributes/PTMs of an expressed product as well. In some cases, specific attributes of the gene product may differ from the fully functional native protein as expressed in healthy persons. Often

AAV gene therapies encode only a portion of the full-length gene due to the size limitation of the capsid delivery vehicle. Differences in activity may result directly from truncation, but post-translational processing also may be affected, making it important to establish the PTM profile of the expressed product *in vivo* to guide development and/or dosing. For example, native Factor VII (FVII) has a highly complex set of PTMs. As observed in studies on expression of recombinant FVII in different tissue types, significant differences in glycan composition result from production in different cells.⁴⁷ Because the site of AAV-derived expression *in vivo* may not perfectly parallel normal endogenous expression in a human patient, differences in the PTM profile may occur and potentially impact clinical efficacy, such as changes in clotting effectiveness, PK, clearance, stability, etc.

In another area, patients with some protein-deficient diseases, such as hemophilia A and B, may be treated effectively at least for some period of time with already marketed naturally purified or recombinant protein therapeutics. A long-term or permanent solution provided by a gene therapy would be preferable, and in that pursuit, there is substantial understanding to be gained from analysis of clinical samples from patients, either with low levels of endogenous protein and/or those treated with protein or enzyme replacement therapies. This analysis would provide a comparison to existing therapeutics in assessing developability and guiding development of gene therapy candidates for these diseases. Importantly, it has been shown that expression of the gene therapy protein product at levels well below normal levels for Factor VIII or IX can result in significant clinical benefit.^{48, 49} indicating these long-lasting gene therapies could actually become a cure for these diseases.

As described herein, carefully designed approaches to MS-based analysis of PTMs from clinical samples can provide detailed, quantitative assessment of attributes and PTMs critical to gene product function and PK/PD to inform

development and guide dosing. The approach of *In Vivo* CQA Mapping involves first establishing robust methods to isolate the protein of interest from clinical samples (serum, tissue) followed by detailed MS-based characterization of the PTM profile through the PK time course to track individual attributes, which approaches are increasingly applied in biologic development today.^{45,46} Quantifying the amounts and persistence of individual attributes and PTMs can also be used to better understand efficacy of a complex, heterogeneous gene therapy expression products. Characterizing CQAs on therapeutic gene therapy products expressed in patients through LC-MS-based *In Vivo* CQA Mapping can identify specific differences from endogenous proteins, elucidate mechanism of action *in vivo* and enable optimization of therapeutic dosing.

Conclusions

The studies and findings summarized in this white paper reflect the tremendous opportunities today in cell and gene therapies and aim to demonstrate the value of applying high-resolution analytical

approaches through development to identify and understand attributes of AAV systems critical to improving efficacy and controlling quality of AAV-based platforms. Additionally, other complex therapies that involve viral and non-viral introduction or modulation of genes can also leverage the information provided by more detailed characterization with MS-based analyses. Characterization using MS and other advanced analytic approaches can similarly support identification of key attributes of each system and further provide opportunities to develop correlations with function and/or outcomes to help improve understanding of their efficacy and reduce safety risks. Importantly, because high-resolution analyses provide detailed mechanistic understanding, these approaches also are increasingly enabling successful platform development in the space going forward.

Acknowledgement

We thank Dr. Rob Garnick and Prof. Barry Karger for strategic input, review of this manuscript and insightful discussion of the topic.

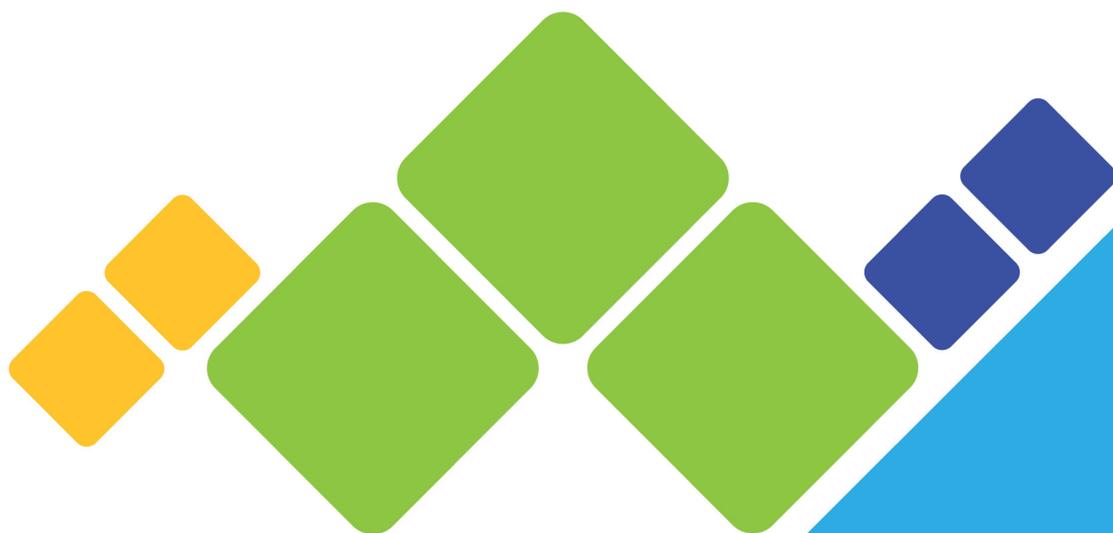
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